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In Vitro Replication of Adeno-Associated Virus DNA: Enhancement by Extracts from Adenovirus-Infected HeLa Cells

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Previously we have described an in vitro assay for the replication of adeno-associated virus type 2 (AAV2) DNA. Addition of the AAV2 nonstructural protein Rep68 to an extract from uninfected cells supports the replication of linear duplex AAV DNA. In this report, we examine replication of linear duplex AAV DNA in extracts from either uninfected or adenovirus (Ad)-infected HeLa cells. The incorporation of radiolabeled nucleotides into full-length linear AAV DNA is 50-fold greater in extracts from Ad-infected cells than in extracts from uninfected cells. In addition, the majority of the labeled full-length AAV DNA molecules synthesized in the Ad-infected extract have two newly replicated strands, whereas the majority of labeled full-length AAV DNA molecules synthesized in the uninfected extract have only one newly replicated strand. The numbers of replication initiations on original templates in the two assays are approximately the same; however, replication in the case of the Ad-infected cell extract is much more likely to result in the synthesis of a full-length AAV DNA molecule. Most of the newly replicated molecules in the assay using uninfected cell extracts are in the form of stem-loop structures. We hypothesize that Ad infection provides a helper function related to elongation during replication by a single-strand displacement mechanism. In the assay using the uninfected HeLa cell extract, replication frequently stalls before reaching the end of the genome, causing the newly synthesized strand to be displaced from the template, with a consequent folding on itself and replication back through the inverted terminal repeat, using itself as a template. In support of this conjecture, replication in the uninfected cell extract of shorter substrate molecules is more efficient, as measured by incorporation of radiolabeled nucleotides into full-length substrate DNA. In addition, when shorter substrate molecules are used as the template in the uninfected HeLa cell assay, a greater proportion of the labeled full-length substrate molecules contain two newly replicated strands. Shorter substrate molecules have no replicative advantage over full-length substrate molecules in the assay using an extract from Ad-infected cells.

Adeno-associated virus (AAV) DNA replication in vivo occurs by a single-strand displacement mechanism similar to that observed for adenovirus (Ad) (3, 9, 10, 24). Figure 1 shows the model for replication starting with linear duplex AAV. The AAV genome contains an inverted terminal repeat (ITR) which is thought to hairpin on itself to serve as the primer to initiate synthesis (9). When the elongating strand has been synthesized to the end of the template, either replication can stop or the newly made strand can fold on itself to initiate a second round of replication. In the latter case, replicative forms would include concatemers of the AAV genome, which have been identified (24). Ends of replicative intermediates are frequently in the hairpinned form, in which the two complementary strands are covalently cross-linked. Eventually, the cross-link is cleaved by the AAV Rep68 or Rep78 protein at a site 124 nucleotides (nt) in from the original 3′ end of the template (12), and the 3′ end of the parental strand is resynthesized, using the transferred hairpin sequence as the template. In this manner, the covalent linkage is dissolved, the original 124 nt at the 3′ end of the parental template strand are transferred to the 5′ end of the progeny strand, and the complete sequence of the ends of the viral genome is maintained (3, 22, 24).

Productive infection by AAV in cell culture normally requires coinfection by a helper virus, usually Ad (1, 3, 16). In the absence of helper virus, AAV gene expression is repressed (2) and viral DNA synthesis is not detectable (20). Most of the Ad helper functions which have been defined are primarily involved with the regulation of gene expression (13, 19, 23, 28). The single exception is E2A (17), which encodes the Ad single-stranded DNA-binding protein; but Ad with a deletion of E2A has been shown to support AAV DNA replication in cell culture with only a twofold reduction compared with wild-type Ad (4). Thus, the available data from in vivo experiments suggest that the failure to detect AAV DNA replication in the absence of Ad coinfection is primarily due to a lack of induction of AAV gene expression.

Two in vitro assays for AAV DNA replication have been described. In the first case, extracts of uninfected HeLa cells supplemented with a maltose-binding protein (MBP)–AAV Rep68 or Rep78 protein fusion protein (6) (or a Rep78 protein expressed from a vaccinia virus vector [15]) can be used to replicate a linear duplex form of AAV DNA or to rescue and replicate an AAV insert in a bacterial plasmid (6, 27). In the second assay, an extract from Ad-infected HeLa cells supplemented with Rep68 or Rep78 expressed from a baculovirus vector is used to replicate a form of duplex AAV DNA in which both strands are covalently cross-linked by terminal hairpin structures (no-end DNA) (18). In the latter assay little if any full-length replication is noted if an extract from uninfected HeLa cells is used.

The results with the second assay suggest that Ad helper function extends beyond simply inducing expression of the AAV Rep proteins. In this report, we describe an apparent Ad helper function related to elongation of the progeny strand

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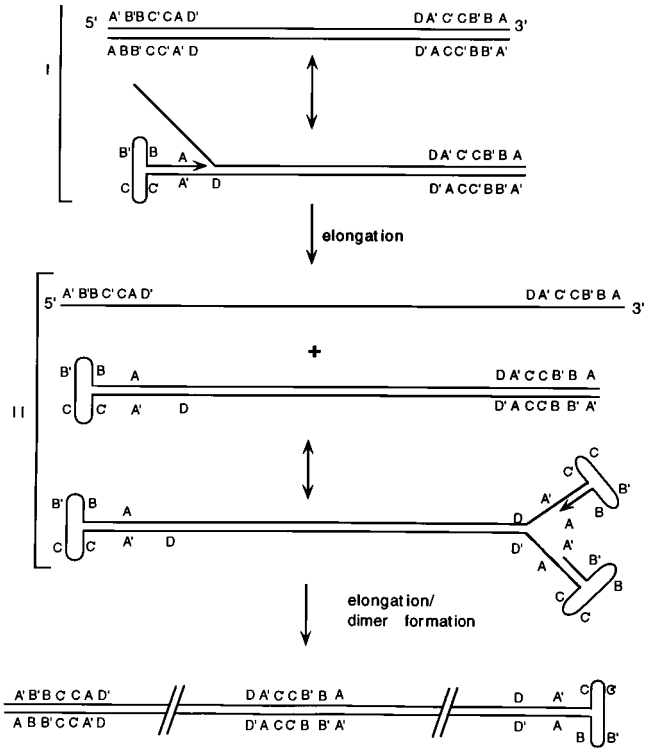

FIG. 1. Model of replication of linear duplex AAV. The 3′ end of one strand folds into a hairpin. This 3′ end serves as a primer for single-stranded displacement producing a linear duplex with one hairpinned end and a displaced single-stranded genome. The newly synthesized 3′ end on the duplex molecule folds over on itself and acts as a primer for continued single-strand displacement synthesis. The result is a dimer molecule which can be resolved into unit-length linear duplexes by Rep endonuclease nicking at the terminal resolution sequence.

during AAV DNA replication. Although use of an extract from uninfected HeLa cells allows synthesis of full-length progeny strands when the initial template is a linear duplex AAV DNA molecule, a majority of the newly synthesized DNA is less than full length. Additionally, there is rarely more than one full-length strand synthesized from any template molecule. We describe experiments which demonstrate that use of an extract from Ad-infected HeLa cells results in greatly increased (50-fold when a linear duplex AAV DNA template is used) synthesis, a majority of full-length progeny strands, and a product that is composed mostly of two newly synthesized strands. These experiments demonstrate that helper functions induced by Ad infection directly affect AAV DNA replication in vitro and therefore seem likely to do so in vivo.

MATERIALS AND METHODS

Preparation of cell extracts. Extracts from uninfected HeLa cells and from HeLa cells infected with Ad were prepared as described previously (11, 25), in a modification of the procedure originally described by Wobbe et al. (29).

AAV Rep68^{MBP} protein. Construction, purification, and characterization of the protein have been described elsewhere (6). It was used in replication as previously described (6, 26, 27).

In vitro DNA replication assay. In vitro DNA replication and analysis of replication products by gel electrophoresis were performed as described previously (27). Products were digested with restriction enzymes according to the manufacturer's instructions except that *Dpn*I digests were performed in 0.210 M NaCl for 3 h (29). PhosphorImager (Molecular Dynamics) scanning of dried gels was performed with Image Quant version 3.0 software. Gels were stained for visualization of total DNA with SYBR Green I (Molecular Probes, Inc., Eugene, Oreg.) according to the manufacturer's instructions.

Plasmids. Plasmid pAV2 has been described elsewhere (14). It consists of the entire genome of AAV2 inserted into a pBR derivative by means of *Bgl*II linkers. The deletion constructs pAV2DD, pAV2DA, pAV2SB, pAV2SS, and pAV2SA were made by digesting pAV2 between the following sites (in parentheses) in AAV: *Dra*III (235)-*Dra*III (3077), *Dra*III (235)-*Apa*I (4045), *Sac*I (810)-*Bst*EII (1700), *Sac*I (810)-*Stu*I (1060), and *Sac*I (810)-*Apa*I (4045), respectively. The ends were then filled in with T4 polymerase, and the plasmids were religated.

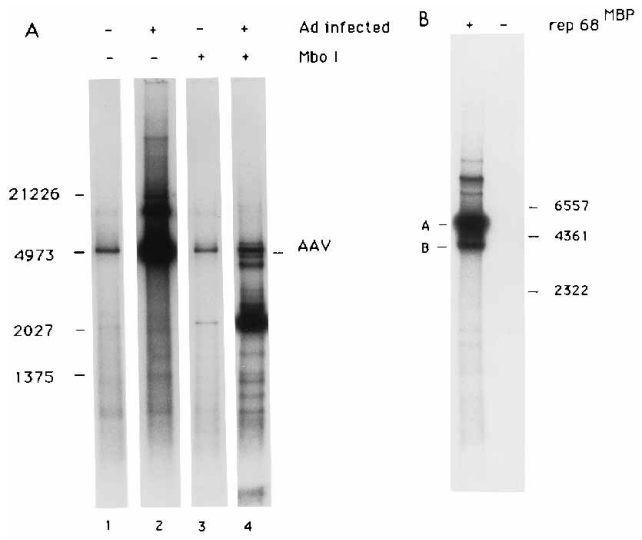

FIG. 2. (A) Replication in assays using an extract either from uninfected HeLa cells or from Ad-infected HeLa cells. Reactions were performed and analyzed as described in Materials and Methods. The substrate in each case is plasmid pAV2 digested with *Bgl*II (which separates the plasmid into linear AAV and pBR molecules) prior to the reaction. Lanes 3 and 4 show, respectively, *Mbo*I digests of the products of the same reactions shown in lanes 1 and 2. Size markers, indicated at the left in nucleotides, were from a *Hin*dIII and *Eco*RI digest of lambda DNA. (B) Replication of pAV2-*Bgl*II in an extract from Ad-infected cells, either with or without the addition of Rep68^{MBP}. Bands labeled A and B both represent full-length AAV, but whereas form A migrates as expected for its length, form B migrates more rapidly in a native gel but migrates at the same rate as form A in an alkaline gel and is presumably an alternative structure (possibly folded in some way). Size markers, indicated at the right in nucleotides, are from a *Hin*dIII digest of lambda DNA.

RESULTS

Extent of replication in Ad-infected and uninfected cell extracts. Ni et al. (18) have shown in their in vitro DNA replication assay with no-end DNA (both ends covalently closed by DNA hairpins) as a substrate little or no replication with the use of an extract made from uninfected cells but substantial replication in an extract made from Ad-infected cells. Previously we showed that in an in vitro assay using extracts from uninfected HeLa cells supplemented with Rep68^{MBP}, linear duplex AAV DNA was an efficient template for DNA replication (26, 27). To determine whether replication with linear duplex AAV templates could be enhanced by the use of Ad-infected extracts, the experiment illustrated in Fig. 2A was performed. The substrate in each of the reactions was plasmid pAV2 (14) digested with *Bgl*II (pAV2-*Bgl*II). pAV2 consists of the entire AAV genome inserted into a pBR derivative by means of *Bgl*II linkers. Therefore, digestion of the plasmid with *Bgl*II produces a linear, duplex AAV fragment and a linear, duplex pBR derivative fragment. There are several nucleotides from the *Bgl*II linker left on the AAV terminus after digestion, but these are apparently removed by some activity present in both the infected and uninfected extracts. Lane 1 illustrates replication in an uninfected cell extract; lane 2 illustrates replication of the same substrate in an extract from Ad-infected cells. In both cases, the AAV fragment replicates whereas the

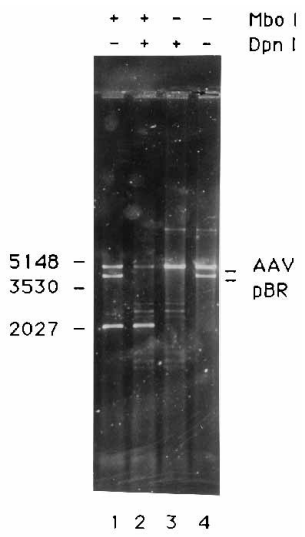

FIG. 3. Replication of pAV2-*Bgl*II in an extract from Ad-infected cells. Replication products in each lane are from the same reaction and were undigested (lane 4) or digested with *Mbo*I (lane 1), *Dpn*I and *Mbo*I (lane 2), or *Dpn*I (lane 3). After electrophoresis, the gel was stained with SYBR Green I (as described in Materials and Methods) and photographed. The largest *Mbo*I digestion product of AAV is visible near size marker 2027. Size markers, indicated at the left in nucleotides, were from a *Hin*dIII and *Eco*RI digest of lambda DNA.

smaller pBR fragment does not. However, significantly more DNA synthesis was observed with the extract of Ad-infected cells; by PhosphorImager analysis, there was a 50-fold increase in the amount of newly synthesized unit-length DNA (compare lane 2 with lane 1).

Figure 2B illustrates two replication assays, both performed in extracts from Ad-infected cells and both with linear duplex AAV as the substrate. One reaction received $Rep68^{MBP}$ and one reaction did not, demonstrating that in extracts from Ad-infected cells, replication of AAV is absolutely dependent on the addition of exogenous Rep68, as was shown previously for uninfected cell extracts (6, 18, 27).

Multiple rounds of replication in an Ad-infected cell extract. The majority of the newly synthesized, full-length strands in an Ad-infected cell extract with linear duplex AAV DNA as the template were susceptible to digestion with *Mbo*I (Fig. 2A, lane 4), in marked contrast to the results obtained with an uninfected cell extract (Fig. 2A, lane 3). Since *Mbo*I digests only DNA with both strands unmethylated, the majority of the double-stranded full-length DNA labeled during the assay contained two newly synthesized strands when the Ad-infected cell extract was used. However, this was clearly not the case when the uninfected cell extract was used. Interestingly, the amounts of labeled DNA resistant to *Mbo*I (hemimethylated DNA, one new strand) were comparable (Fig. 2A, lanes 3 and 4).

To visualize all of the DNA in the reaction using an Ad-infected cell extract, reaction products were stained with SYBR Green I after gel electrophoresis (Fig. 3). The doublet at the position of unit-length DNA represents AAV (slower) and vector (faster) DNA. As expected, the pBR322 vector was totally susceptible to *Dpn*I, whereas a majority of the AAV DNA was resistant to *Dpn*I, and susceptible to *Mbo*I. Thus, again it is possible to conclude that a majority of the newly synthesized full-length DNA was in double helices containing two new strands. A comparison of the relative amounts of DNA in the AAV and pBR322 bands (lane 4) indicates that there is approximately three times more AAV DNA than pBR322. Since the original reaction contained equal amounts of AAV and pBR322, produced by digestion of pAV2 with *Bgl*II, the data indicate net synthesis of AAV DNA in the assay. By calculating the fraction of the unit-length AAV DNA susceptible to *Mbo*I (about 1.5×) and the fraction of the remainder resistant to *Dpn*I (about 0.75×), the fraction of unreplicated AAV DNA after the reaction was about 0.75×. This result indicates that only about 0.25× of the original AAV DNA molecules was replicated and that an initial round of replication of a molecule greatly increased the probability of subsequent rounds of replication of that molecule. These data support the data in Fig. 2 that Ad-infected extracts lead to net synthesis of AAV DNA and that those parental molecules which are replicated undergo more than one round of synthesis. Both of these results are different from the results observed for an uninfected cell extract.

Aberrant replication products. Another difference observed is that an Ad-infected cell extract causes synthesis of a majority of full-length strands, whereas the uninfected cell extract produces a majority of newly synthesized strands which are less than full length (as illustrated in Fig. 4, which is a PhosphorImager tracing of two such experiments). The nature of the products in the broad low-molecular-weight smear was determined by performing various digestions of the material and analyzing the products on two-dimensional gels (data not shown). The results were confirmed by cloning one of the smallest species, which is shown in Fig. 5. There are two aberrent forms: a single-stranded form which is a stem-loop structure with the stem from nt 1 to 350 and the loop from nt 351 to 760, and a double-stranded form which is simply the duplex form of the single-stranded structure. Replication starts at the ITR and proceeds by single-stranded displacement. At some point, e.g., nt 760, replication stalls, the newly elongated strand is displaced from the template and folds on itself at a complementary sequence (in this case nt 760 to 350), and replication continues back to the ITR, using the displaced new strand as the template. The product is a stem-loop structure; the double-stranded form can be produced by replicating the stem-loop form. Presumably, the different lengths of the aberrant molecules reflect different points at which the elongating strand came off the template. Despite the fact that the absolute amount of total synthesis is 50-fold higher in the Ad-infected cell extract, the absolute amount of aberrant product in the Ad-infected extract is more than 4-fold lower (in the experiment shown in Fig. 4) and reflects yet another difference from the reaction using an uninfected cell extract.

Extent of initiation on parental molecules. Data in Fig. 2, which showed that the amounts of hemimethylated material were comparable in uninfected and Ad-infected extracts, and in Fig. 3, which showed that the extensive replication seen in the Ad-infected extract was due to multiple rounds of replication initiating on about one-quarter of the input substrate molecules, suggested that the numbers of initiations in the two extracts might be similar. To determine directly the extent to which the difference in overall amount of synthesis observed reflected a difference in the fraction of input AAV DNA molecules on which replication initiated, the following experiment was performed. A DNA substrate containing only the left ITR was created by digesting the linear, duplex AAV DNA with *Sna*BI (which cuts at nt 4496), removing the right ITR. With this substrate, use of an Ad-infected cell extract led to a 7-fold-greater amount of DNA synthesis (compared with the 50-fold difference seen with substrates which had both the left and right ITRs). This value, i.e., sevenfold, represented both the greater processivity with an Ad-infected cell extract and any potential increase in the frequency of initiation on the input

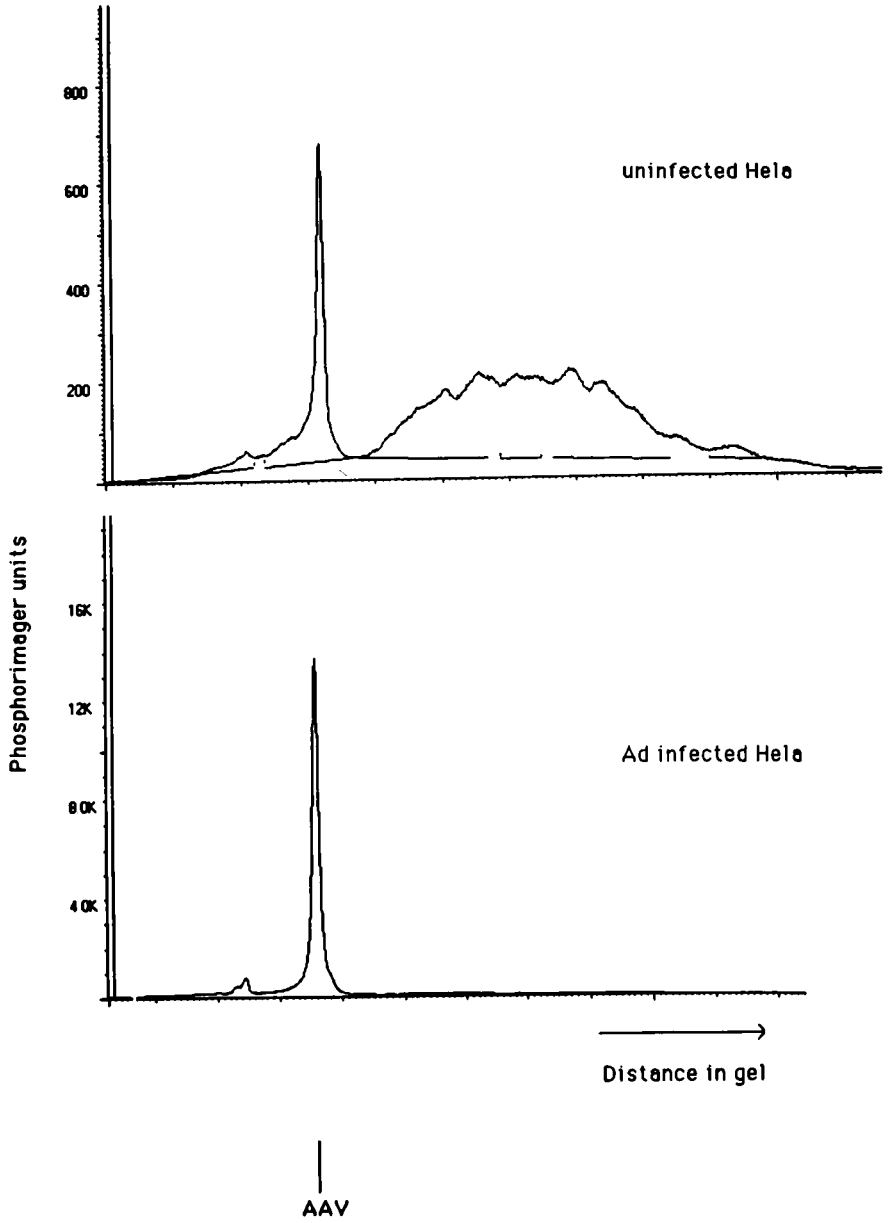

FIG. 4. PhosphorImager tracing of two lanes from agarose gels comparing replication of pAV2-*Bgl*II in an extract from uninfected cells with that in an extract from Ad-infected cells. The area under the curve is proportional to the radioactivity on that part of the lane. The horizontal axis represents distance on the gel, with higher-molecular-weight material on the left and lower-molecular-weight material on the right. The peak representing full-length linear duplex AAV is indicated. The vertical axis indicates PhosphorImager units showing relative radioactivity.

molecules. To measure incorporation adjacent to the origin, i.e., the left ITR, and thereby minimize the difference due to processivity, the *Sna*BI replicated product was digested with *Sac*II, which cuts AAV DNA at nt 682 and 2411. A comparison of the relative levels of incorporation in each of the three *Sac*II bands in each of the reactions is shown in Fig. 6B. A comparison of replication through the first 682 nt now shows less than a twofold difference by PhosphorImager analysis and indicates that even in this assay, the majority of the increase in DNA synthesis seen with the Ad extract is most likely caused by greater processivity and not by increased initiation of synthesis on the original AAV DNA.

Use of shorter substrate molecules. Because the major difference observed with the two extracts with linear duplex DNA as the substrate seemed to involve processivity, we performed a series of replication assays using uninfected cell extracts with equimolar amounts of AAV DNA substrates of various lengths. All had the ITR and original adjacent sequences at both ends, with various amounts of internal sequences deleted. Figure 7 contains a diagram illustrating five of the shorter DNA substrates relative to full-length duplex DNA and gel electrophoresis of the products of reactions containing these substrates. The quantitative relationship between length and incorporation of radioactivity is shown in Fig. 8. Replication of the shortest substrate (800 bp) led to incorporation of 40 times the radioactivity incorporated with full-length DNA as the substrate. Given the difference in genome length, this is the equivalent of 240 times the number of genome equivalents. In an assay in which the right ITR was removed from the shortest substrate, the increase in DNA synthesis completely disappears (data not shown); thus, the difference again was not due to a significantly greater frequency of initiation.

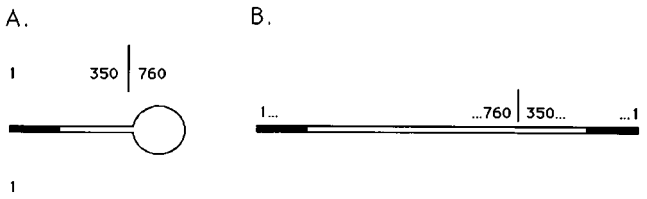

FIG. 5. Diagram showing the two forms of one species of aberrant product. The numbers designate the AAV base number at the transition point in this species. A and B represent, respectively, the single-stranded stem-loop form and the double-stranded linear form of the aberrant product.

Comparison of the extents of replication of a longer and a shorter substrate molecule in an Ad-infected extract. If the enhancement of replication from the use of shorter substrates reflects less displacement of the elongating strand from the template and if absence of displacement is the primary mechanism of enhancement by Ad extracts, then in Ad-infected extracts, shorter molecules should replicate no better than long molecules. The result of such a comparison is shown in Fig. 9A, in which replication of a shorter molecule (the substrate of lane 5 in Fig. 7) by an Ad-infected extract was equivalent, by PhosphorImager analysis, to that seen for a full-length AAV molecule in a parallel reaction. Since the shorter molecule is less than one-half as long as the longer mole-cule, this means that the shorter molecule must have replicated more than twice as many times as the longer molecule for equivalent amounts of synthesis. There is an upper limit for total incorporation in this assay which limits how many rounds of replication each substrate molecule can undergo.

Additionally, replication of a shorter molecule (the substrate of lane 5 in Fig. 7) was examined in Ad-infected and uninfected extracts. The replication advantage for the shorter substrate in the Ad-infected cell extract was only 4-fold, compared with 50-fold for full-length AAV, by PhosphorImager analysis (Fig. 9B).

As observed with the products of the increased replication of full-length DNA by use of the Ad-infected extract, the products of the increased synthesis seen in assays using the shorter DNAs with an uninfected cell extract were increasingly duplex molecules in which both strands were newly synthesized. Figure 9C shows the two substrates of Fig. 9A, replicated in an uninfected cell extract digested with *Mbo*I. In each case the high-molecular-weight band is the *Mbo*I-resistant substrate and the lower-molecular-weight band (band m) is the largest *Mbo*I digestion product. The shorter substrate led to more product with two newly synthesized strands susceptible to *Mbo*I.

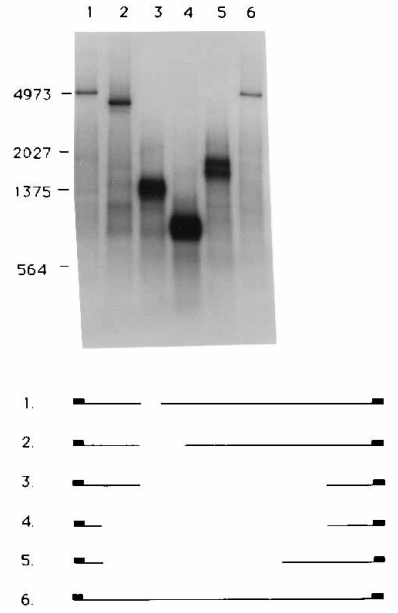

FIG. 7. Comparison of replication of full-length linear duplex AAV with replication of linear duplex deletion mutants of AAV in an extract from uninfected cells. Shown are the products of replication of equimolar amounts of wild-type AAV and the five deletion mutants described in Materials and Methods (lane 1, pAV2SS; lane 2, pAV2SB; lane 3, pAV2SA; lane 4, pAV2DA; lane 5, pAV2DD; lane 6, pAV2). The lane numbers correspond to the deletion mutants shown in the diagram. Size markers, indicated at the left in nucleotides, were from a *Hin*dIII and *Eco*RI digest of lambda DNA.

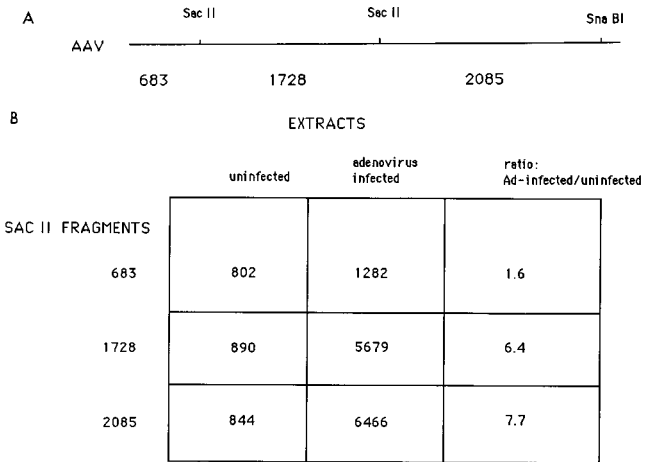

FIG. 6. Comparison of replication of a substrate containing only one ITR in uninfected and Ad-infected cell extracts. Replication was performed on linear duplex AAV as described in Materials and Methods except that prior to replication, the right ITR was removed from the substrate by *Sna*BI digestion. After replication, the replication products were digested with *Sac*II and separated by gel electrophoresis, and incorporation into each band was determined by PhosphorImager analysis. (A) *Sac*II-*Sna*BI restriction map of AAV; (B) relative incorporation into each band in the two reactions.

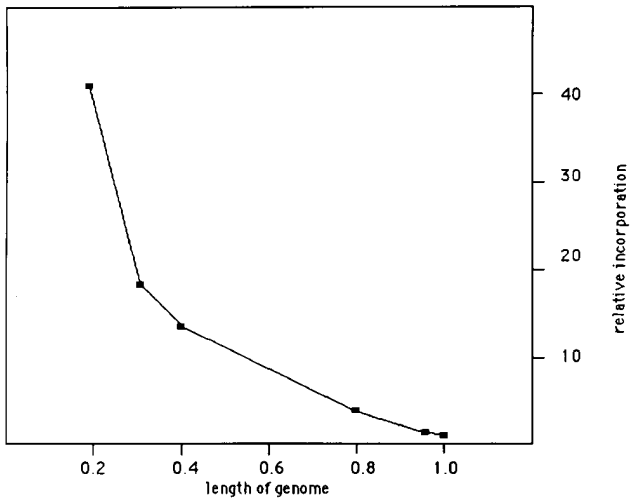

FIG. 8. Graph of incorporation versus length of substrate, showing the amount of incorporation of radioactive nucleotides into each of the *Bgl*II-digested plasmids used for Fig. 6 after replication performed with equimolar amounts of digested plasmid. The horizontal axis shows the length of the deletion mutants relative to that of wild-type AAV. The vertical axis shows relative incorporation, with the incorporation of the wild type, i.e., pAV2, arbitrarily set at 1.0.

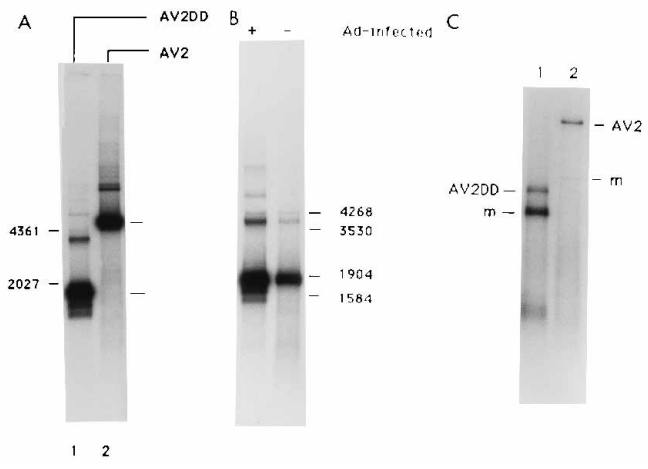

FIG. 9. (A) Replication of the full-length substrate and of a shorter substrate in an extract from Ad-infected cells. Equimolar amounts of pAV2DD-*Bgl*II (lane 1) and pAV2-*Bgl*II (lane 2) were replicated as described in Materials and Methods in an extract from Ad-infected cells. Size markers, indicated on the left in nucleotides, were from a *Hin*dIII digest of lambda DNA. (B) Replication of a shorter substrate (pAV2DD-*Bgl*II) in an extract from Ad-infected cells and in an extract from uninfected cells. Size markers indicated on the right were from an *Eco*RI-*Hin*dIII digest of lambda DNA. (C) *Mbo*I digestion of a deletion mutant replication. Replication of equimolar amounts of a deletion mutant (pAV2DD-*Bgl*II; lane 1) and full-length AAV (pAV2-*Bgl*II; lane 2) in an extract from uninfected HeLa cells was followed by *Mbo*I digestion prior to gel electrophoresis. In each lane, the uppermost band represents undigested material and the next-largest band (designated m) represents the largest *Mbo*I fragment from a digestion of the replicated substrate.

DISCUSSION

A general principle of AAV biology is that AAV does not replicate in cell culture unless the cells are coinfected by a helper virus. Whether AAV DNA replication requires the helper virus only to stimulate the synthesis of Rep proteins or whether additional Ad functions, directly involved in DNA replication, are needed has been unclear. The in vitro system of Ni et al. (18), in which exogenous Rep proteins were insufficient to promote DNA synthesis in an extract from uninfected cells but did promote replication in an extract from Ad-infected cells, demonstrated that in vitro (and by extrapolation in vivo) the Ad helper was providing additional replication functions.

Previously we reported replication in which exogenous Rep proteins were able to cause synthesis of full-length AAV DNA in an extract from uninfected cells (6, 27). This report, which demonstrates a greatly enhanced replication in extracts from Ad-infected cells, supports the conclusion of Ni et al. (18) and in addition, by comparing levels of replication in the two extracts, delineates one Ad helper function.

Assays using substrates with only one ITR show that in this assay, the numbers of AAV DNA replication initiations on the original substrate molecules in the extract from Ad cells and in the extract from uninfected cells are not substantially different. While there might be the potential for higher levels of initiation in Ad-infected cells, in the in vitro assay, higher levels of initiation do not explain the observed enhancement. The increased DNA synthesis in the Ad-infected cell extract must be the result of increased synthesis per initiation of a parental molecule. This could be due to greater processivity and/or greater ability to switch template strands at the end of one round of replication to begin a subsequent round of replication. As discussed below, the increased DNA synthesis is apparently due to greater processivity. We have no evidence for an increased ability to switch template strands at the end of one round of replication in the Ad-infected cell extract, but increased processivity in the Ad-infected cell extracts means that the replication complex is much more likely to complete a round of replication and be in a position to switch template strands and commence an additional round of replication.

There is a substantial difference in processivity between the two extracts. Substrates with one ITR, which showed approximately equivalent incorporation for the region adjacent to the ITR, showed a sevenfold-greater incorporation in the extract from Ad-infected cells when incorporation into the full 4,500-base length of the one ITR substrate was measured. This failure of processivity, as measured by lesser amounts of incorporation at points in the substrate more distant from the origin of replication, seems to correlate with the presence of the products of an aberrant replication mechanism. We hypothesize that in extracts from uninfected cells, replication stalls at various points. The consequence of this interruption in replication is the displacement of the newly synthesized strand from the double helix of the substrate DNA. Once displaced from the template, the single-stranded molecule pairs with itself at some region of limited homology and replicates back through the ITR, using itself as the substrate, with the resultant formation of a stem-loop structure.

It seems likely that this phenomenon might be the origin of the genomes found in defective interfering (DI) particles as described by Hauswirth and Berns (10), de la Maza and Carter (7), and Senapathy and Carter (21). The description of DI particles, internally deleted but with normal ITR sequences, corresponds to the molecules described in this report. The production of these aberrant molecules does occur in assays using extracts from Ad-infected cells, but the absolute amount is less than one-fourth as much as in an assay using an extract from uninfected cells despite the much more extensive replication in the former extract. Assays using a mix of extracts from uninfected and Ad-infected cells give rise to a substantial quantity of these aberrant products. In coinfected cells, DI genomes accumulate preferentially early in the AAV replicative cycle, which, in light of the results described here, might be expected if the cells had not yet been made fully permissive for AAV elongation (5).

Discussions of DI particles have sought to explain the stem-loop structures seen by electron microscopy in one of two ways. One explanation is that the newly made strand is displaced, folds back on itself, and continues replicating, using itself as a template (10). The second explanation is that the displaced strand from the original template breaks and then folds back on itself, forming a potential primer-template structure for subsequent elongation of the broken strand (8). The predictions arising from the two models are quite different. The results of this work, showing *Mbo*I sensitivity of the product and that often the majority of radioactive incorporation is into stem-loop structures, suggest that in vitro the stem-loop structures originate in the newly synthesized strand. The implication is that the DI particles seen in vivo also result from displacement of the newly synthesized strand.

A second substantial difference between replication in the extract from Ad-infected cells and replication in the extract from uninfected cells is that in the former case, most of the unit-length labeled DNA has both strands replicated, compared with the latter case, in which most unit-length labeled DNA contains only one newly synthesized strand. Staining of the total DNA from a reaction with a DNA-binding stain and digestion with *Mbo*I and *Dpn*I allow comparisons of the relative numbers of molecules which are unreplicated, replicated on one strand, and replicated on both strands. The data show

that approximately 75% of the input molecules are unreplicated. Thus, to account for the large quantity of molecules which are replicated on both strands and the considerable increase in total amount of DNA, a subset of molecules must have replicated numerous times. This replication of only some of the input molecules is not unexpected, given that the capacity for initiation on the original parental molecules does not seem much higher in the extract from infected cells. The implication is that second and subsequent rounds of replication are more likely on molecules which have already replicated. Apparently it is not the case that replication, at the completion of one round, automatically proceeds into replication of the second strand. Assays performed with larger amounts of substrate DNA lead to lower percentages of *Mbo*I-sensitive replication products, suggesting that the commencement of replication of the second strand is in competition with initiation of replication of the first strand on a different molecule (26a). Rather, when the factors required for replication are in excess, second-round replication seems to involve an initiation process that is more likely on molecules which have already replicated than on those which have not replicated.

To explore further the failure of extracts from uninfected cells to support a second round of replication, we performed assays with shorter substrates. Shorter substrates showed an increased ability to undergo a second round of replication. In addition, these shorter substrates gave greater amounts of net synthesis. The increased net synthesis is most likely the result of undergoing more than one round of replication, as shown by the increasing susceptibility to *Mbo*I digestion of the replication product when shorter substrates were used. The conclusion is that the extracts from uninfected cells are quite able to support the folding of the ITR and the template strand switch by the polymerase which are the requirements for a second round of replication.

The requirement for the helper function supplied by the Ad-infected extract can be overcome by the use of a shorter template molecule. This seems likely to be the consequence of two factors. The first would be that there is less probability of the displacement of the elongating strand when the template is shorter. This seems to be primarily a function of length and not of the deletion of specific sequences, although it is likely that some sequences might predispose more to displacement of the growing strand. Possibly of more interest is that initiation of a second round of replication apparently restarts the clock with respect to displacement; hence, with a short enough template, multiple rounds of synthesis of full-length copies of the template occur even in an uninfected cell extract. With respect to the last point, it is important to note that the number of rounds of replication in the assays using the short molecules in the uninfected cell extract was approximately five times greater than seen when full-length molecules were replicated in the extract from Ad-infected cells. Even when the shortest templates were used, which led to significant net synthesis, only a fraction of the original DNA molecules were replicated. We suspect that this inability to replicate all original substrate molecules reflects a structural variability which we have not yet defined.

In conclusion, in vitro AAV DNA replication is substantially greater in an assay which uses an extract from Ad-infected cells than in one using an extract from uninfected cells, and this enhancement is principally due to a greater processivity of replication in the infected cell extract. This is not a surprising finding, since the ability to carry out extensive single-strand displacement replication is not an expected property of eukaryotic cells.

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